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14. ABSTRACT This proposal is focused on the illustration of PrLZ, a novel prostate-specific gene identified in our laboratory. In the first year of a three-year project, we have prepared most of the molecular constructs necessary for the proposed experiments. We have determined that over-expression of this protein indeed promotes accelerated growth of prostate cancer cells. The underlying mechanism seems to be through interaction with the 14-3-3 proteins, especially the 14-3-3σ protein. Progress made in these studies lays a solid basis for continued research of PrLZ, an important regulatory gene in prostate morphogenesis, and in the development of prostate cancer.					
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INTRODUCTION

The objective of this research proposal is to elucidate the biological function of the PrLZ, a novel prostate-specific gene identified in our laboratory. In the funded proposal, we hypothesized that the prostate-specific leucine zipper protein PrLZ promoted proliferation of the prostate epithelial cells. Abnormal expression of the PrLZ may be involved in the development of prostate cancer. Specific aims of the proposal were to determine the causal relationship between PrLZ expression and prostate cancer progression, to determine the effect of 14-3-3 binding on the function of the PrLZ, and to define midkine as a downstream target gene of the PrLZ. We proposed first to change expression of the PrLZ in prostate cancer cell lines to confirm the growth-promoting function. At the same time, the mechanism of function will be elucidated by examining the interacting proteins with the PrLZ. Accordingly, we proposed four research tasks, which ensure the completion of these studies in a three-year research project. In the past year, our commitment has led to marked progress of the project as scheduled.

BODY

In the following sections, research activities and progression of the project will be described in detail, in accordance to the Statement of Work that was approved by the granting agency.

Task 1. Prepare expression constructs for functional analysis of the PrLZ.

a. Prepare and confirm DNA constructs for PrLZ over-expression in pcDNA3.1(-).

As reported previously, we have cloned a full-length coding sequence for the PrLZ. We then realized that messenger of the PrLZ was alternatively spliced, and a total of five PrLZ isoforms have been recognized in the prostate cancer cell line, LNCaP (Appendix 1). We cloned all these isoforms with RT-PCR assisted cloning. DNA sequencing analysis was used to confirm their sequence identity. Subsequently, all the five isoforms were transferred to pcDNA3.1(-), a mammalian expression vector.

b. Design and develop siRNA expression cassettes for knocking down PrLZ expression.

PrLZ is a member of an expanding TPD52 gene family. It shares sequence identity with other members of the family. In order to select siRNA that specifically knocked down the PrLZ, we designed two siRNA sequences, based on the coding sequence unique to the PrLZ. In transient transfections, we found that both of the sequences could inhibit 70% of the PrLZ protein production in the C4-2 cells, as determined by western blotting. We suspected that the incomplete knockdown was due to limited efficiency of the transfection.

c. Clone the siRNA to the mammalian expression vector in pSECneo.

Both of the siRNA are currently been cloned into the siRNA expression vectors, which will be stably transfected to the C4-2 cells. It is our hope that the C4-2 clones with stable transfection of these constructs will have better inhibition of the PrLZ expression.

d. Construct and confirm the PrLZ bait plasmids in pCMV-AD.

This part of the project has been completed. We have cloned all the five cDNA clones for the five PrLZ isoforms, to the pACT and the pBIND vectors (Promega), respectively. Availability was the reason for using these vectors of the mammalian two-hybrid system over the pCMV-AD,

which was proposed originally in the application. DNA sequences of the constructs were confirmed and the constructs have been used in the mammalian two-hybrid assays to detect interaction of the PrLZ proteins with members of the 14-3-3 protein family. The result of the mammalian two-hybrid will be reported in the later section (Appendix 1).

e. Construct and confirm the 14-3-3 target plasmids in pCMV-BD.

This part of the project has been completed. Using RT-PCR assisted cloning and DNA sequencing, we have cloned seven cDNA clones representing all the seven known 14-3-3 genes in humans. Inserts from these clones were then transferred to the pACT and the pBIND vectors, respectively. DNA sequences of the constructs were again confirmed and the constructs have been used in the mammalian two-hybrid assays to detect interaction of the PrLZ proteins with members of the 14-3-3 protein family. The result of the mammalian two-hybrid will be reported in the later section (Appendix 1).

f. Construct and confirm the construct for inducible expression of the PrLZ in pTRE-tight.

The cDNA inserts representing the five PrLZ isoforms were transferred to a modified pTRE-tight vector. Because the commercial pTRE-tight vector does not contain any antibiotic selection marker, we modified the original vector by introducing a coding sequence of the hygromycin resistant gene. The purpose of this modification was to facilitate the stable double transfection of the regulator plasmid, the pTetOn, in combination with the response vector containing the PrLZ cDNA inserts (Figure 1).

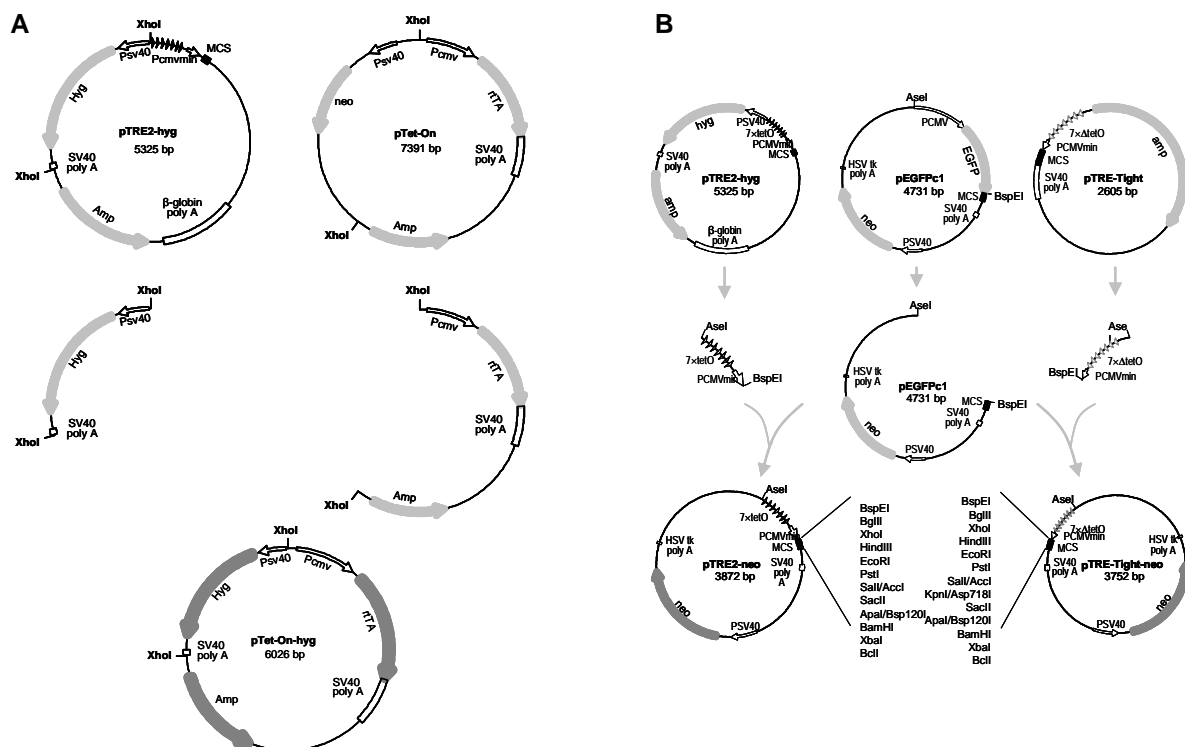


Figure 1. Modifying vectors of the inducible expression system to facilitate isolation of stably transfected PrLZ clones. We modified the tetracycline inducible system (Clontech). The original pTetOn vector was changed to carry a hygromycin resistant marker (A), and the pTRE-tight vector was added with a neo marker (B). With these modifications, inducible expression of the PrLZ could be studied in stably transfected cells and could be used in xenograft inoculations. Function of the modified vectors were confirmed (see Figure 2).

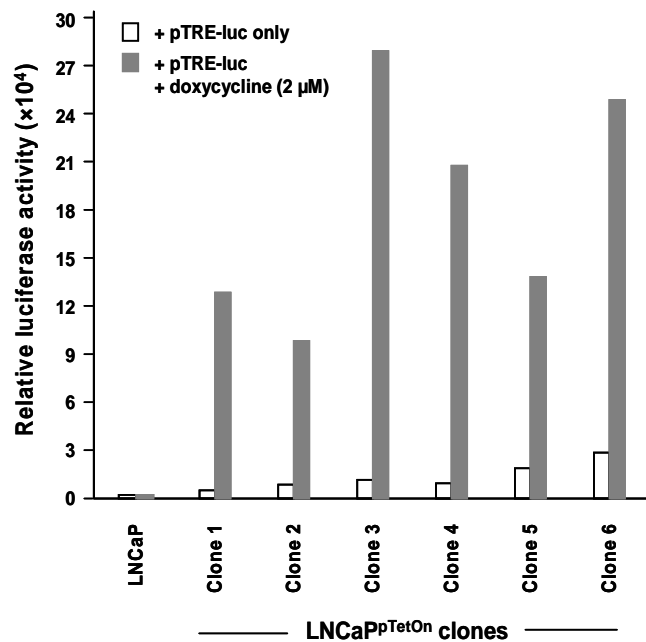


Figure 2. Verification of the established LNCaP clones with inducible expression. LNCaP cells were transfected with the pTetOn vector and stable clones selected. These clones were subjected to transient transfection with the pTRE-luc plasmid, and 48 hours later, were treated with doxycycline. Cells were harvested 24 hours after the treatment and were examined for luciferase activity. The data presented were mean from triplicate measure. For all presented data, standard deviations were less than 5% of the mean and were not shown.

At the same time, we established cell lines, in which the newly constructed vectors could be used. LNCaP cells were transfected with commercial pTetOn regulatory vector and stable clones were isolated. By transiently transfecting these clones with the pTRE-luc responsive plasmid and treating the cells with doxycycline, we identified several highly inducible LNCaP clones, yielding more than 30 times higher luciferase activities upon a doxycycline treatment, as compared to those without the doxycycline treatment (Figure 2).

Currently, the LNCaP clones with the highest inducible response by doxycycline is been transfected with the modified pTRE-tight harboring the PrLZ coding sequences. Hygromycin B is used to select the transfected clones. These clones containing the dual DNA constructs are expected to respond to the doxycycline induction, manifesting with a doxycycline-dependent

expression of the PrLZ protein, which will be confirmed with western blotting analysis. The confirmed clones will be used in the proposed studies.

Task 2. Prepare cell lines for functional analysis of the PrLZ.

a. Isolate LNCaP cell clones over-expressing the PrLZ.

We have isolated the LNCaP clones that over-expressing one of the PrLZ isoforms. The over-expression was confirmed with western blot analysis (Figure 3).

We have isolated LNCaP clones over-expressing the other four isoforms. Western blottings are used to identify the the LNCaP clones with substantially elevated expression of the PrLZ isoforms.

b. Isolate C4-2 cell clones with repressed PrLZ expression.

This task has not been completed. At the moment, siRNA sequences used to knock down the PrLZ are been cloned to the expression vector. The constructed vector will then be stably transfected to the C4-2 cells. It is our estimate that C4-2 clones with repressed PrLZ expression may be identified in another few months.

c. Isolate LNCaP cell clones stably transfected with the inducible constructs.

This task has not been completed. Presently, the LNCaP clones with the highest inducibility is been transfected with the modified pTRE-tight harboring the PrLZ coding sequences. Hygromycin B is used to select the transfected clones. Next, the selected clones have to be treated with doxycycline, followed by western blotting to confirm the inducible expression of the PrLZ. We expect that these works could be completed in six months.

Task 3. Characterizing the function of the PrLZ.

a. Study tumorigenicity of the PrLZ by subcutaneous implanting LNCaP cells over-expressing the PrLZ.

We have completed one of these experiments. LNCaP cells were forced to express high levels of one of the isoforms of the PrLZ. Three of the clones were subcutaneously inoculated to the hormonally intact Balb/c^{nu/nu} mice. All the three groups (n=6) mice showed enhanced tumor growth, in contrast to the control group.

We are currently performing similar experiments on castrated animals to determine whether over-expression of the PrLZ endows these cells the capability of androgen-independent growth. It is known that LNCaP cells, when grown in xenograft, are androgen dependent. If the PrLZ functions to promote cell growth and survival, LNCaP cells over-expressing the PrLZ protein should grow to form tumors, in the absence of male hormone.

b. Study metastatic potential of the PrLZ by intra-cardiac injection of LNCaP cells over-expressing the PrLZ.

We plan to start these experiments in the new year.

c. Study tumorigenicity of the PrLZ by subcutaneous implanting C4-2 cells with repressed PrLZ expression.

These experiments rely on the establishment of stably isolated C4-2 cell lines with the PrLZ expression knocked down. We are currently cloning the siRNA to an expression vector. C4-2 cells will be transfected with the construct and selected for stable transcription of the siRNA. Three selected clones will be used in this part of the study.

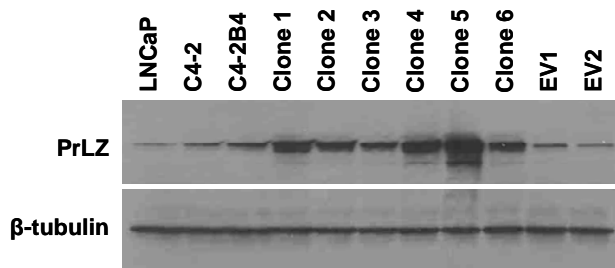


Figure 3. Identification of PrLZ-overexpressing clones.

In this experiment, LNCaP cells were transfected with the longest isoform of the PrLZ (PrLZ264) in pCDNA3.1(-). Following selection with G418 (400 µg/ml for 3 weeks), 24 individual clones were screened for PrLZ expression with western blotting. In the upper panel, 6 clones with high levels of PrLZ expression were re-confirmed with western blotting. The EV1 and EV2 represent two clones transfected with the pCDNA3.1(-) empty vector.

d. Study metastatic potential of the PrLZ by intra-cardiac injection of C4-2 cells with repressed PrLZ expression.

These experiments rely on the establishment of stably isolated C4-2 cell lines with the PrLZ expression knocked down. We are currently cloning the siRNA to an expression vector. C4-2 cells will be transfected with the construct and selected for stable transcription of the siRNA. Three selected clones will be used in this part of the study.

e. Identify the PrLZ-binding 14-3-3 isoform by mammalian two-hybrid assay.

Part of this study has been carried out and reported. In this study, we first cloned all the five PrLZ isoforms into the mammalian two-hybrid vectors, and the coding sequences for all the seven known 14-3-3 genes into the same vectors. Secondly, we combined each of the PrLZ/pACT constructs with each of the 14-3-3/pBIND constructs in mammalian two-hybrid assay, by co-transfecting the combined plasmids with a luciferase reporter construct (pG5luc) into the C4-2 cells.

Alternatively, we also combined each of the PrLZ/pBIND constructs with each of the 14-3-3/pACT constructs. An interaction of the PrLZ and 14-3-3 proteins was indicated with increased luciferase activity in the cells. These series of studies indicated that, even though PrLZ may interact with several 14-3-3 proteins, it has a more intimate interaction specifically with the 14-3-3 σ , which is also known as the Stratifin (SFN) protein (Appendix 1).

We are now repeating the mammalian-two hybrid assays in the C4-2 cells and the LNCaP cells, and also in the HEK293 cells. The use of these cell lines is necessary because they contain different levels of endogenous PrLZ and 14-3-3 proteins. By comparing the results of the same mammalian-two hybrid assays in different cells, we may be able to better identify the interacting isoforms of the PrLZ with the 14-3-3 proteins. These assays may also identify one of these cell lines, with which the physical interaction of the PrLZ and 14-3-3 could be conclusively determined with the method of co-immunoprecipitation.

f. Determine the relationship between PrLZ and midkine expression by inducible PrLZ expression.

As reported in the preceding section, we have transfected the pTetOn into LNCaP cells and selected clones that are highly responsive to doxycycline induction (more than 30 times). PrLZ coding sequences were transferred to the pTRE-tight vector, which we have engineered to contain a hygromycin resistant marker. We are presently selecting the doubly transfected clones that may exhibit inducible PrLZ expression.

The resultant LNCaP clones will be treated with doxycyclin to induce expression of the PrLZ. Subsequently, the treated cells will be examined for altered midkine expression, with RT-PCR and western blotting. Our preliminary data showed that expression of the midkine was enhanced in the LNCaP clones over-expressing the PrLZ. Using the inducible PrLZ, we may confirm this finding.

We have cloned a 2.3 kb genomic region that contained the promoter region of the midkine gene, by PCR amplification and DNA sequencing analysis. This promoter will be used to control a coding sequence of the luciferase reporter. This construct will facilitate further study on the regulation of midkine expression by the PrLZ.

Task 4. Analysis of the xenograft tumors and preparation of reports.

a. Perform statistical analysis of the xenograft results.

This part of the project has not been completed.

b. Perform immunohistochemical analysis of the xenograft PCa tumors.

This part of the project has not been completed.

c. Examine for altered osteo-mimicry markers.

This part of the project has not been completed.

d. Study the association of PrLZ-binding 14-3-3 isoform with PCa progression.

This part of the project has not been completed, even though we have primarily identified the interacting 14-3-3 protein in the C4-2 cells.

e. Prepare reports for publication.

We have reported part of this project as poster presentations:

KEY RESEARCH ACCOMPLISHMENTS:

- We have completed most part of the task 1.
- We have completed most part of the task 2
- We have completed the preliminary study of the task 3.

REPORTABLE OUTCOMES

1. In the past year, we have successfully verified all the DNA constructs needed for the mammalian two-hybrid assay.
2. A novel tetracycline inducible vector is created, with antibiotic selection markers. This vector could be used in dual selection for stable cells containing two expressional plasmids.
3. A panels of LNCaP and C4-2 cell clones was characterized for their stable expression of the transfected genes.
4. PrLZ has been confirmed to interact with the 14-3-3 proteins.

CONCLUSION

In the past year, we have successfully completed most of the proposed studies as proposed. It is our expectation that the continued work will provide us with concrete data, supporting that the PrLZ is a growth-associated prostate-specific protein, and it does so by interacting and thus modulating the function of other growth-related genes.

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AG Baseman, AJ Kirsch, FF Marshall, **HE Zhau**, LWK Chung, and **RX Wang** (2005). 14-3-3 expression patterns in the human kidney: from fetal development to the adult state to malignant

transformation. *Americical Urological Association, 100th Annual Meeting, San Antonio, TX, May 21-26, 2005. #378.*

RX Wang, JC Xu, T Miyagi, N Mabjeesh, MJ Fox, M Amin, FF Marshall, **HE Zhau**, and LWK Chung (2006). PrLZ, oncogenic property implicated by its expression patterns in normal development and tumor progression of the human prostate. (*manuscript in preparation*).

APPENDICES

1. JC Xu, H Fu, FF Marshall, HE Zhau, LWK Chung, and R Wang. The 14-3-3 proteins: expression in prostate epithelia and interaction with the novel and prostate-specific PrLZ protein. Poster. (See attachment.)

SUPPORTING DATA

None.



The 14-3-3 proteins: expression in prostate epithelia and interaction with the novel and prostate-specific PrLZ protein.

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Abstract

Introduction and Objectives. We have reported the characterization of a novel and prostate-specific gene, PrLZ (Prostate Leucine Zipper), whose levels of expression were correlated with proliferative activity of the prostate epithelia, both in prostate morphogenesis and in prostate cancer progression. The PrLZ contains a conserved IRSIQHSIMPAMR domain. Upon phosphorylation of the central serines, this domain becomes a binding site by 14-3-3 proteins, which are known to interact with regulatory proteins to modulate cell survival and proliferation. We investigated the interaction between 14-3-3 and the PrLZ proteins in the prostate cancer cell lines.

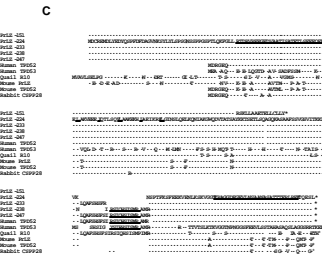
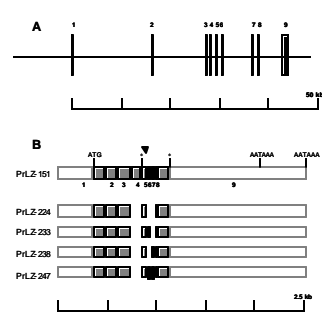
Methods. Prostate cancer cell lines used in this study included the LNCaP and its lineage derived subclones of C4-2 and C4-2B, PC3, PC3M, DU145, and ARCaP. Semi-quantitative RT-PCR was used to examine expressions of the 14-3-3 genes. Samples of the normal prostates were used as controls for comparison. Mammalian two-hybrid system was used to detect the interaction between PrLZ and the 14-3-3 proteins.

Results. We determined the expression of all the seven known 14-3-3 genes in normal prostate samples. Expression of the 14-3-3 genes was then compared between normal prostates and the prostate cancer cell lines. We observed no changes in the expressions of the 14-3-3 α , 14-3-3 ϵ , and 14-3-3 γ genes. Expression of the 14-3-3 ζ was increased in all the prostate cancer cell lines compared to that of the normal prostate, while expressions of the 14-3-3 η , 14-3-3 σ , and 14-3-3 τ genes were decreased. In the LNCaP lineage, loss of expression of the 14-3-3 σ and 14-3-3 τ genes was correlated to the progressive malignant potentials of these cells. Mammalian two-hybrid assays indicated possible interactions between the PrLZ and these 14-3-3 proteins.

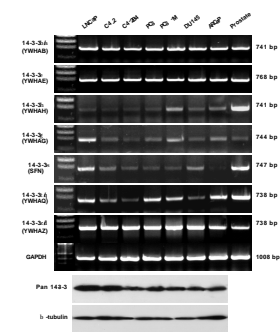
Conclusions. We assessed function of the PrLZ by examining its interaction with 14-3-3 proteins. An inverse relationship between the enhanced PrLZ expression and the decreased 14-3-3 σ and 14-3-3 τ was found. It is known that 14-3-3 proteins sequester important regulatory proteins to inhibit their nuclear function. In the prostate, growth-promoting property of the PrLZ may be modulated through its interaction with 14-3-3 proteins. In prostate cancer, loss of the 14-3-3 σ and 14-3-3 τ may lead to an uncontrolled PrLZ activity, which in turn promotes proliferation of the cancer cells.

Results

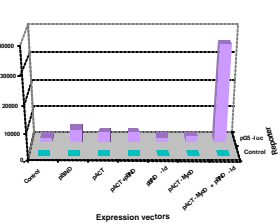
PrLZ variants derived from alternative splicing



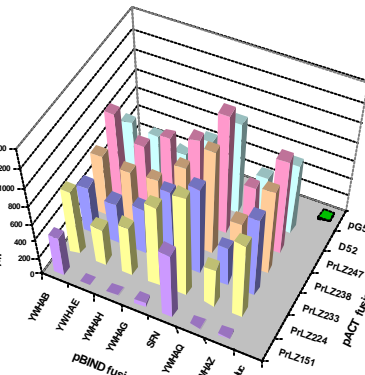
Differentiated expression of 14-3-3 genes



Mammalian two hybrid system in C4-2 cells



Interaction between PrLZ variants and 14-3-3 proteins



Conclusions

1. Among the 7 known 14-3-3 genes, 14-3-3 β , ϵ , and ζ are constitutively expressed in prostate cancer cell lines, at levels similar to those of the normal prostate, while the other 14-3-3 genes are differentially expressed.
2. In addition to the canonical 14-3-3 binding domain, other regions of the PrLZ are involved in interaction with the 14-3-3 proteins.
3. Mutational mapping is needed to map these regions.